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Antihepatotoxic effect of ethanolic extract of *Pellionia heyneana* Wedd., leaf on paracetamol induced liver damage in Wistar rats

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Abstract

Pellionia heyneana Wedd. leaf has been used by the Cholanaikan tribe as traditional medicine to enhance general health, immunity and also to treat various liver ailments. However, no scientific reports are available regarding its traditional claim. In the present study, scientific evaluation of antihepatotoxic activity of *P. heyneana* crude ethanolic leaf extract (PHLE) was carried out on paracetamol induced liver damage in Wistar rats. The substantially altered serum parameters were restored towards normalization significantly by the plant extract. The biochemical observations were supplemented with histopathological examinations of liver samples. All these results are comparable with the known hepatoprotective agent, silymarin.

Keywords: Cholanaikan tribe, *Pellionia heyneana* Wedd., Paracetamol, Hepatoprotection.

1. Introduction

Pellionia heyneana Wedd., is an erect or decumbent herb of Urticaceae family with 15 to 25 cm long stem with creeping base, frequently growing in moist evergreen forests (Fig. 1). The plant is found distributed in Peninsular India, Sri Lanka, Cambodia, Indonesia, Thailand, and China. Cholanaikan are the most primitive, vanishing and diminutive tribal community of Kerala State. They are also known as the most 'Primitive cavemen' of Kerala (Mathur, 2013) inhabiting the evergreen interior tropical rain-fed forest of the upper valley of the Nilambur forest, Malappuram District, Western Ghats region. Cholanaikan call this plant as 'Elaven'. The medicinal use of this plant was recorded first time during the ethnobiological studies carried out by S. Rajasekharan and his team during the year 1989. It is a rare information disclosed by Shri. Kuppamala Kaniyan aged 85 years, who was residing in the interior forest area of Nilambur. According to him, the expressed juice of the fresh plant is administered in children as an immuno-enhancer and also rejuvenate the functions of vital organs like liver, heart and kidney (Rajasekharan and Nair, 1994).

Fig. 1: *Pellionia heyneana* Wedd.

Based on the above information, the plant has been selected to scientifically validate the antihepatotoxic activity of *P. heyneana* against paracetamol induced hepatotoxicity in Wistar rats.

2. Materials and Methods

2.1 Collection and authentication of plant material

P. heyneana plants were collected from Kallar, Thiruvananthapuram district of Kerala, India, (Fig 2) and authenticated by the plant taxonomist of the Institute. Voucher specimens were deposited at the Jawaharlal Nehru Tropical Botanic Garden and Research Institute Herbarium (TBGT 57060 dated 10/12/2010).

Fig. 2: Author Collecting information on *Pellionia heyneana*

2.2 Preparation of extract of *P. heyneana* leaf

P. heyneana leaves were collected, shade dried and powdered. The powder was extracted with 95% ethanol for 48 h, using soxhlet apparatus. The extract was then filtered and the filtrate was concentrated under reduced pressure using rotary evaporator, to get the crude extract. This crude extract was referred to as PHLE.

2.3 Animals

Wistar rats (150-200 g) were obtained from the Institute's Animal House. All the animals were housed in polypropylene cages under standard conditions at temperature 25 ± 2°C, relative humidity 60 ± 10%, room air changes 15 ± 3 times/h and 12 h light-dark cycles, fed commercial rat feed (Lipton India Ltd; Mumbai, India) and boiled water *ad libitum*. Animals were acclimatized for 1 week before the initiation of an experiment. The study was carried out according to National Institute of Health (NIH) guidelines, after getting the approval of the Institute's Animal Ethics Committee

2.4 Commercial kits

Commercial kits for the estimation of Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), γ-Glutamyl transferase (GGT), Serum bilirubin (SB),Triglycerides (TGL), Total cholesterol (TC) and Total protein (TP) were purchased from Coral Clinical System, Goa, India.

2.5 Evaluation of *in vitro* antioxidant activity

In vitro antioxidant activity of PHLE was evaluated using standard procedures as mentioned below.

2.5.1 Estimation of DPPH radical scavenging activity

DPPH radical scavenging activity of PHLE was assayed using the standard method (Blois,1958). A methanolic solution of (2 mL) of DPPH (0.025 g/L) was added to 200 µL of the different concentrations (25 µg/mL to 200 µg/mL) of PHLE and allowed to react at room temperature for 30 min in dark and the absorbance was measured at 517 nm. Methanol served as the blank and 200 µL of methanol was added to DPPH in positive control tubes, instead of plant extract. Ascorbic acid was used as the standard.

$$\text{Percentage of inhibition} = \frac{\text{Absb*.of Control - Absb.of Sample}}{\text{Absorbance of Control}} \times 100$$

*Absorbance

From the calculated DPPH radical scavenging activity, the EC_{50} was calculated, which represents the concentration of the scavenging compound that caused 50% neutralization.

2.5.2 Estimation of nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of PHLE was measured using the standard procedure (Mondal *et al*., 2006). Sodium nitroprusside (1mL of 10 mM) was mixed with 1 mL of different concentration (25 to 200 µg/mL) of plant extract in phosphate buffer (pH 7.4). The mixture was incubated at 25˚C for 150 min. To 1 mL of the incubated solution, 1 mL of Griess' reagent (1% Sulphanilamide, 2% O-phosphoric acid and 1% Napthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546 nm and percentage of inhibition was calculated using the formula (1). Ascorbic acid was used as standard.

2.5.3 Anti-lipid peroxidation studies

The anti-lipid peroxidation effects of PHLE was studied *in vitro*, following the modified method (Suja *et al*., 2003a). Briefly, 0.5 g of the rat liver tissue was sliced and homogenized with 10 mL of 150 mm Kcl-Tris-HCl- Buffer (pH 7.2). The reaction mixture was composed of 0.25 mL of liver homogenate, 0.1 mL Tris-HCl buffer (pH 7.2), 0.05 mL ascorbic acid (AA) (0.1 mm), 0.05 $FeCl_2$ (4 mm) and 0.05 mL of various concentrations of plant extract. The mixture was incubated at 37º C for 1h in capped tubes. Then 0.5 mL of 0.1 N HCl, 0.2 mL of 9.8% sodium dodecyl dulphate (SDS), 0.9 mL of distilled water and 2 mL of 0.6% thio barbituric acid (TBA) were added to each tube and vigorously shaken. The tubes were placed in a boiling water bath at 100ºC for 30 min. After cooling, the flocculent precipitate was removed by adding 5 mL of n - butanol and centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 532 nm.

2.6 Paracetamol (APAP) induced hepatotoxicity

Paracetamol induced hepatotoxicity was carried out according to the procedure of Suja *et al.* (2003b). Wistar rats were divided into six groups (6 animals/ group), Groups I and II were the normal control and paracetamol intoxicated group respectively and both received a single daily dose of 0.5% Tween-80 (1 mL, p.o.) for all 6 days. Group III, IV and V were administered PHLE reconstituted in 0.5% Tween- 80 at dosages (100, 200 and 300 mg/kg, p. o.) for all 6 days and Group VI was administered Silymarin, the standard hepatoprotective drug, at a dose of 100 mg/kg, p.o., for all 6 days. Paracetamol suspension (2.5 g/kg, p.o.) was administered to Groups II to VI on 5th day, 30 min after plant extract/silymarin administration. On the 6th day after 24 h starvation, all the animals were sacrificed by Carbondioxide inhalation. The collected blood was allowed to coagulate for 1h at room temperature. It was centrifuged at 1500 rpm for 15 min at 37ºC to separate the serum. The serum was then used for biochemical estimations. Liver samples collected in PBS were subjected to antioxidant assays (estimation of Malondialdehyde (MDA), assay of Catalase (CAT), determination of Reduced glutathione (GSH)) and liver slices preserved in 10% neutral buffered formaldehyde solution were subjected to histopathological examination.

2.6.1 Biochemical estimations

The collected serum was subjected to the assay of hepatic marker enzymes, namely Aspartate

transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), γ-Glutamyl transferase and other serum parameters namely, Serum Bilirubin (SB), Total Cholesterol (TC) and Triglycerides (TGL) using commercial kits by Coral Clinical System, Goa, India.

2.6.2 Estimation of liver malondialdehyde

Malondialdehyde (MDA) in the rat liver was estimated by the modified procedure of Ohkawa *et al.* (1979). Liver homogenate (10% w/v) from each group (1 mL) mixed with 100 µL of 8.1% SDS and 600 µL of 20% acetic acid solution was kept for 2 min at room temperature. Then 600 µL of 0.8% solution of TBA was added, heated at 95ºC for 60 min in water bath and cooled with ice cold water at 4ºC. A mixture of n-butanol and pyridine (15:1 v/v) were added, shaken vigorously and centrifuged at 10,000 rpm for 5 min. The absorbance of the organic layer was measured at 532 nm. Lipid peroxidation was expressed as nmol/g wet liver.

2.6.3 Assay of liver catalase

Catalase (CAT) in the rat liver was assayed according to the method of Aebi (1974). To 0.9 mL of phosphate buffer (0.01M, pH-7.0) 0.1mL of liver homogenate (10% w/v) and 0.4mL of H_2O_2 (0.2 M) were added. After 60 sec, 2 mL of dichromate - acetic acid reagent (5%) was added. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620 nm. Standard H_2O_2 in the range of 2-10 µL was taken with blank containing reagent alone. CAT activity was measured proportionately to the rate of H_2O_2 reduction. Dichromate in acetic acid was converted to perchromic acid and then chromic acetate, when heated in the presence of H_2O_2. Chromic acetate formed was measured at 620 nm. Absorbance values were compared with a standard curve generated from known catalase and the activities were expressed as U/mg protein. Amount of protein/mg of tissue was determined by the standard method (Lowry *et al*., 1951).

2.6.4 Estimation of liver reduced glutathione

Reduced glutathione (GSH) in the rat liver was assayed according to the method of Ellman (1959). Homogenized rat liver samples (10% w/v) from the entire group (0.2 mL) was mixed with 1.8 mL of 1 mM EDTA solution. To this, 3.0 mL precipitating reagent (1.67 g of metaphosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride and 1L distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2 mL of the supernatant, 4.0 mL of 0.3 M disodium hydrogen phosphate solution and 1.0 mL of DTNB (5, 5'dithio-bis (2-nitro benzoic acid)) reagent were added and absorbance was read at 412 nm. Absorbance values were compared with a standard curve generated from known GSH.

2.6.5 Histopathological investigations

Liver slices preserved in formalin solution were subjected to dehydration with acetone of strength 70, 80 and 100 % respectively, each for 1 h. Infiltration and impregnation were done by treatment with paraffin wax, twice each time for 1 h. Paraffin was used to prepare paraffin 'L' moulds. Specimens were cut into sections of 3-7 µm thickness and stained with haematoxylin and eosin. The thin sections of the liver were made in to permanent slides and examined under high resolution microscope with photographic facility and photomicrographs were taken.

2.7 Statistical analysis

All the data were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). The significance of difference among the group was assessed using one way analysis of variance (ANOVA) followed by Dunnett's post-test using SPSS software version-20. The $p \leq 0.05$ was considered statistically significant.

3. Results and discussion

3.1 DPPH radical scavenging activity

PHLE showed potent antiradical activity by inhibiting DPPH radical in a dose dependent manner (Fig. 3). Maximum DPPH radical scavenging (65.94%)was observed at 200µg/mL and the EC_{50} was found to be 82.38 µg/mL.

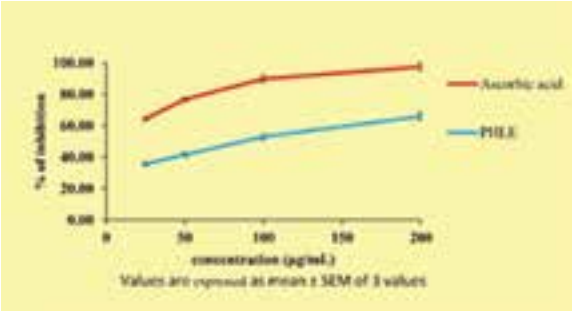

Fig. 3: DPPH radical scavenging activity of *Pellionia heyneana* leaf ethanolic extract in comparison with ascorbic acid standard

3.2 Nitric oxide radical scavenging activity

The results of NO radical scavenging activity of PHLE was represented as percentage of NO scavenging in Fig. 4. The nitric oxide radical scavenging activity PHLE was found to be increasing as their concentration increased from 25 µg/mL to 250 µg/mL. PHLE showed maximum nitric oxide radical scavenging of 70.26% at 200 µg/mL and the EC_{50} of the extract was found to be 64.30 µg/mL.

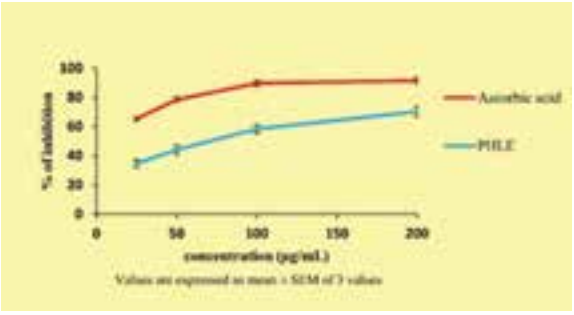

Fig. 4: Nitric oxide radical scavenging activity of *Pellionia heyneana* leaf ethanolic extract in comparison with ascorbic acid standard

3.3 *In vitro* anti-lipid peroxidation studies

The extract showed potent inhibition of $FeCl_2$-AA stimulated rat liver lipid peroxidation in dose dependent manner (Table 1). The degree of lipid

Table: 1. Inhibitory effect of *Pellionia heyneana* extract (PHLE) on $FeCl_2$- Ascorbic acid (AA) induced lipid peroxidation in rat liver homogenate *in vitro*.

Group	Drug concentration (µg/mL)	MDA (nmol/g wet liver)	MDA inhibition (%)	EC_{50} (µg/mL)
Normal control	-	0.78 ± 0.04	-	-
Toxin control ($FeCl_2$-AA)	-	2.31 ± 0.14	-	-
$FeCl_2$-AA + PHLE	25	1.28 ± 0.12	44.59 ± 4.18	59.88
$FeCl_2$-AA + PHLE	50	1.16 ± 0.13	49.78 ± 4.68	
$FeCl_2$-AA + PHLE	100	1.14 ± 0.08	50.64 ± 3.42	
$FeCl_2$-AA + PHLE	200	0.94 ± 0.07*	59.31 ± 3.34	

Values are expressed as mean ± SD of 3 values, one way ANOVA followed by Dunnett's multiple comparison test, *$p \leq 0.05$ compared to toxin control.

peroxidation in the liver tissue was determined in terms of malondialdehyde (MDA) produced as nmol/g wet liver. A significant increase of MDA has observed in $FeCl_2$-AA treated rat liver homogenate, compared to normal control without $FeCl_2$-AA. Treatment with PHLE showed a maximum % of inhibition in MDA production 59.31 ± 3.34 at 200 µg/mL dose compared to toxin control. The EC_{50} values of *in vitro* anti-lipid peroxidation in terms of MDA inhibition % of PHLE was found to be 59.88 µg/mL.

3.4 Paracetamol (APAP) induced hepatotoxicity

Paracetamol or acetaminophen (APAP) is the most commonly sold over-the counter antipyretic analgesic, which is generally considered harmless at therapeutic doses. However, APAP overdose causes severe and sometimes fatal hepatic damage in humans and experimental animals (Davidson and Eastham, 1966; Mitchell, 1988; Sumioka *et al*., 2004). At therapeutic doses, about 80% of paracetamol is conjugated directly to form sulfate and glucuronide esters before oxidation and are excreted in bile or urine. During metabolism, small amount of APAP is converted in to N-acetyl-p-benzoquinone-imine (NAPQI), a highly reactive metabolic intermediate by cytochrome P450 (CYP) (Dahlin *et al*., 1984), which is normally detoxified by conjugation with GSH. High doses of APAP results in irreversible conjugation of more NAPQI with GSH and when hepatic GSH is depleted, more NAPQI will bind covalently to cellular macromolecules (Jollow *et al*., 1973, Potter and Hinson, 1986) and ultimately leads to cell death. Upon the administration of APAP, mice and hamsters have been shown to develop fulminant centrilobular necrosis similar to that observed in humans (Davis *et al*., 1974; Potter *et al*., 1974). Based on this background, liver toxicity induction model using APAP was adopted to check the efficacy of the plant extract and fraction of *P. heyneana* to combat liver diseases.

In the present investigation, it was observed that APAP administration causes a complex mechanistic sequence of events as mentioned above which results in the leakage of liver enzymes into blood serum. A significant ($p \leq 0.05$) increase in the level of serum marker enzymes namely AST, ALT, ALP and GGT in APAP alone administered groups as compared with the normal control. Interestingly, PHLE administered groups showed a significant decrease in the liver enzyme levels to some extent in a dose dependent manner, similar to the standard hepatoprotective drug Silymarin treated group. This may be attributed to the hepatoprotective activity offered by the administration of the *P. heyneana* against APAP induced liver toxicity.

Increased levels of serum bilirubin in APAP alone administered group may be due to the excessive haeme destruction and blockage of the biliary tract, mass inhibition of the conjugation reaction or release of unconjugated bilirubin from damaged and dead hepatocytes as previously reported by Wolf *et al*. (1997). *P. heyneana* extract administered groups showed significantly lower bilirubin levels compared to the toxin group which reveals the ability of the plant extract to bring the liver function into normal.

Serum level of TC and TGL were also significantly increased in paracetamol treated rats, when compared to control (Table 2).

Table: 2. Effect of *Pellionia heyneana* leaf ethanolic extract (PHLE) on serum markers of hepatic injury in APAP intoxicated Wistar rats

Treatment groups	Parameters							
	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	GGT (IU/L)	SB (mg/dL)	TC (mg/dL)	TGL (mg/dL)	TP (g/dL)
Normal control	91.64±1.29	59.23±3.63	96.73±1.12	6.82±1.23	0.34±0.04	98.66±1.54	116.45±3.65	5.93±0.32
APAP (2.5 g/kg)	263.41±3.95*	218.45±3.64*	274.76±3.54*	24.84±2.42	1.58±0.21*	196.28±3.49	236.47±3.13*	3.75±0.12*
APAP+PHLE (100 mg/kg)	157.46±1.25**	160.15±3.18**	179.50±2.17**	13.4±2.54**	0.72±0.06**	131.26±2.45**	157.47±2.54**	4.28±0.10**
APAP+PHLE (200 mg/kg)	147.5±1.81**	88.35±2.94**	155.35±2.92**	12.94±1.64**	0.68±0.13**	115.47±3.45**	151.95±1.64**	4.74±0.12**
APAP+PHLE (300 mg/kg)	136.48±3.29**	76.55±2.50**	141.87±3.27**	8.25±0.85**	0.61±0.05**	106.34±3.68**	139.64±1.70**	5.17±0.19**
APAP+Silymarin (100 mg/kg)	109.54±2.42**	63.77±0.34**	120.73±2.03**	7.39±0.72**	0.52±0.06**	100.75±2.25**	121.05±1.22**	5.69±0.17**

Values are expressed as mean ± SEM of six values, one way ANOVA followed by Dunnett's multiple comparison test,*$p \leq 0.05$ compared to normal control, **$p \leq 0.05$ compared to APAP control.

Elevated serum levels of TC and TGL in APAP administered groups of this experiment also indicates impaired fat metabolism due to hepatic damage. Administration of *P. heyneana* leaf extract and fraction significantly decreased serum lipid profile in paracetamol toxicity induced rats. From this evidence, it can be hypothesized that the plant extract restored the fat metabolism and the extracts also possess hypolipidemic activity which is evident from the significant reduction in TC and TGL in plant drug treated groups compared to toxin control group. Healthy functioning of liver is required for the synthesis of serum proteins except for the γ-globulins. The decreased TP in toxin control group may be due to decreased protein metabolism in the liver and is a feature of liver damage (Kanchana and Sadiq, 2011). Interestingly, the significant increase in the serum protein levels in the entire plant drug treated groups also supports the hepatoprotective nature of *P. heyneana*.

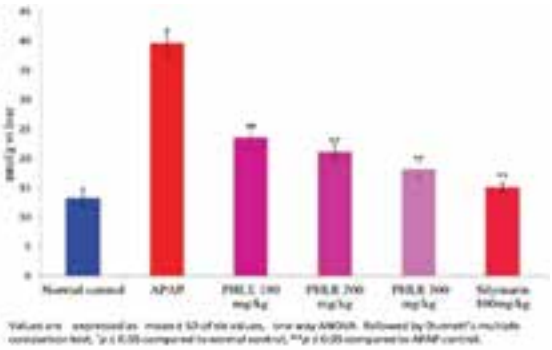

Fig 5 : Effect of *Pellionia heyneana* leaf ethanolic extract on liver MDA after APAP administration

Results obtained in the present investigation showed an increase in the level of MDA and a decrease of GSH and catalase level in the APAP administered groups (Fig. 5, 6 & 7). These findings were well agreement with the previous studies in this line in which an increase level of MDA (Slattery *et al*., 1987) decreased level of GSH (Saito *et al*., 2010) and CAT (Rajkapoor *et al*., 2008) are very obvious in paracetamol induced liver toxicity.

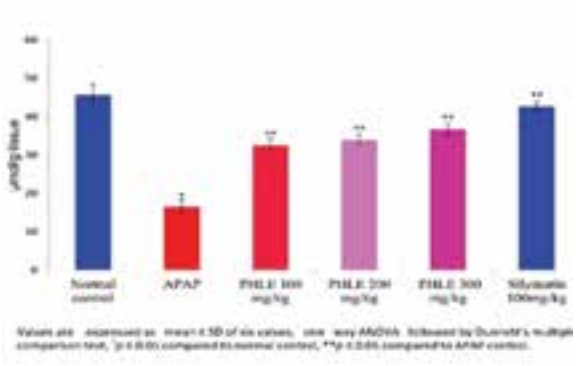

Fig 6 : Effect of *Pellionia heyneana* leaf ethanolic extract (PHLE) on liver GSH after APAP administration

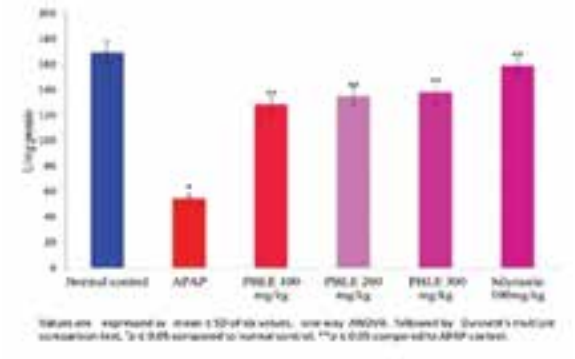

Fig 7 : Effect of *Pellionia heyneana* leaf ethanolic extract (PHLE) on liver CAT after APAP administration

The plant extract administered groups ameliorated these changes by causing a decrease in the level of MDA and an increase in the level of GSH and CAT in a dose dependent manner.

3.5 Histopathological investigations

The histopathological profile of control animals showed normal hepatic architecture. Liver section of APAP administered toxic groups showed a remarkable variation from the control group with intensive liver injuries. The liver damage induced by the administration of APAP was found to be reduced considerably in PHLE and silymarin treated groups (Fig. 8). Histopathological findings also support the biochemical observations of the present study and confirms the curative effect of PHLE against APAP induced liver damage.

4. Conclusion

The findings of the present study have thus brought out scientific evidence as to the profound efficacy of *P. heyneana* for curing liver disorders by the synergetic action of different mechanisms including radical scavenging, enhancing the level of both enzymatic and non-enzymatic antioxidants and keeping the integrity of hepatocytes through anti lipid peroxidation. Over all, this study provides novel insights in to the therapeutic potential of *P. heyneana* as a hepatoprotective candidate against paracetamol induced hepatotoxicity.

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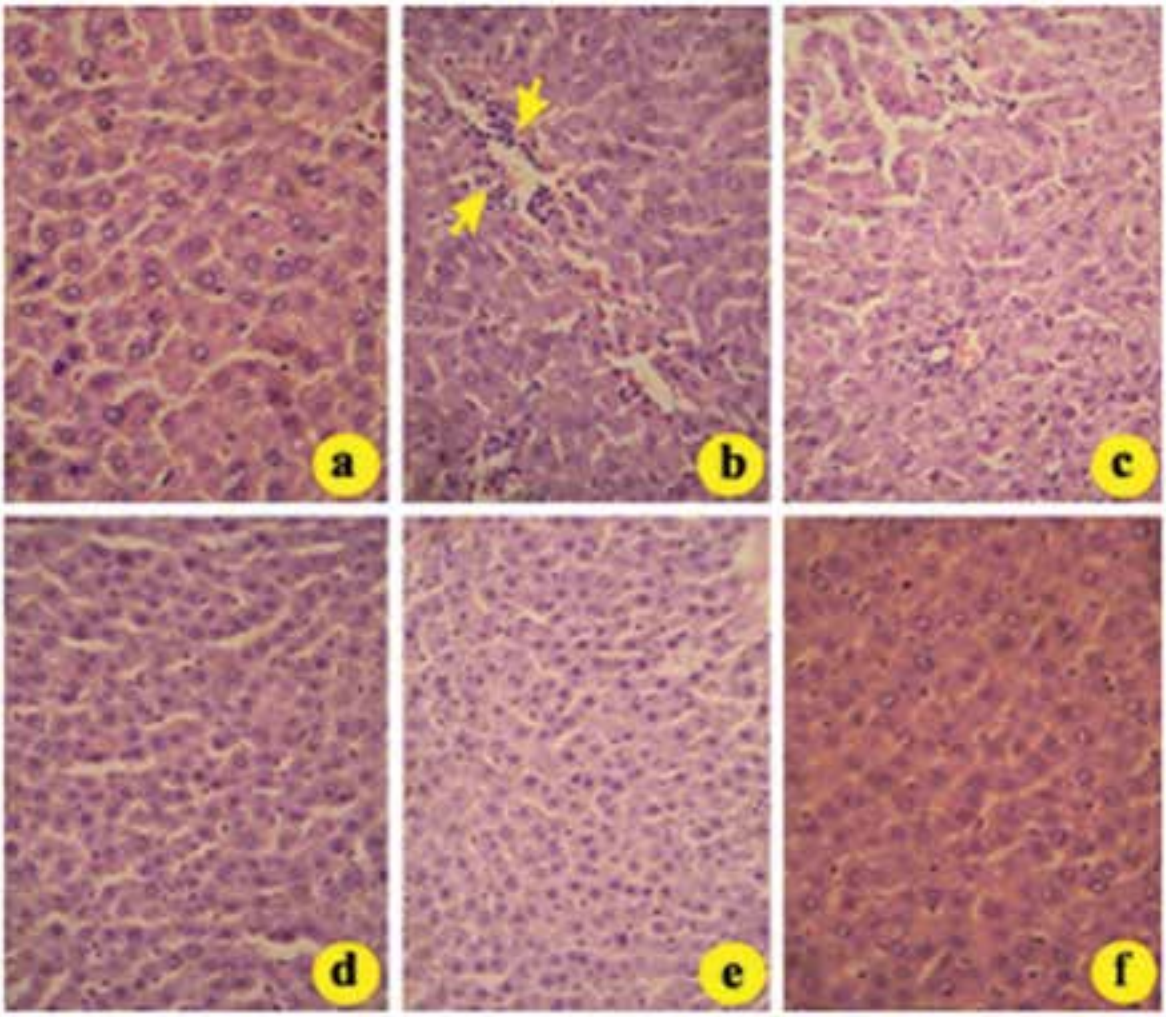

Fig. 8: Histopathological features of APAP induced hepatotoxicity in Wistar rats. (a) Control rat liver showing the portal triad and the normal hepatocytes with well brought out nuclei and cytoplasm. (b) APAP treated rat liver showing centrilobular necrosis, loss of cell boundaries and ballooning degenerations,(c) (d) & (e) are liver sections of rat treated with PHLE (100mg/kg b. wt., 200 mg/kg b. wt. and 300mg/kg b. wt. respectively) showing minimal hepatic damage. (f) Silymarin treated rat liver section showing almost normal hepatic architecture. (Magnification-X600)

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